A severe and a mild potato spindle tuber viroid isolate differ in three nucleotide exchanges only

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Fingerprint analyses of two potato spindle tuber viroid (PSTV) isolates causing severe and mild symptoms, respectively, in tomato exhibited defined differences in the RNase T₁ and RNase A fingerprints. The complete sequencing of the mild isolate and the comparison of its primary structure with the previously established one of the pathogenic type strain revealed that oligonucleotides CAAAAAAG, CUUUUUUCUAUCUUACUUG, and AAAAAAGGAC in the 'severe' strain are replaced by CAAUAAG, CUUUUUUCUAUCUUUCUUUG, AAU, and AAGGAC in the 'mild' strain. Thus, three nucleotide exchanges at different sites of the molecule may change a pathogenic viroid to a practically non-pathogenic isolate. The possible correlation between the secondary structure in a defined region of the PSTV molecule and its pathogenicity for tomato is discussed.

Viroids are unique plant pathogens and at present about nine different viroids are known as the causal agents of economically important plant diseases (1-3). Viroids differ from conventional viruses in that their small single-stranded circular RNA with a molecular weight of about 120 000 (4) is not protected by a capsid protein. The complete molecular structure of the potato spindle tuber viroid (PSTV) has been established (5), and in its native state the 359 ribonucleotides of the PSTV molecule were shown to form a largely double-stranded rod-like structure which is characterized by an alternating arrangement of short base-paired and unpaired regions (6). These structural features are characteristic of all viroids studied so far (7).

By comparison, almost nothing is known about the mechanism and the molecular basis of viroid pathogenicity. Viroids are apparently the only pathogens that do not code for any protein (8-10), and they seem to contain the information for their own structure only. Fingerprinting techniques have demonstrated that PSTV, citrus exocortis viroid (CEV), and chrysanthemum stunt viroid (CSV) are all distinctly different RNA species with marked differences in their primary sequence (11-12). Even strains of PSTV which differ in pathogenicity.

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have been differentiated by their RNA fingerprints (13). Since RNA is the only known component of viroids, these differences in nucleotide sequence can be related to differences in viroid host range and pathogenicity. Consequently, a comparison between their primary sequences will allow deeper insights into the interrelations between the structure of viroids and their biological functions. In this study we wish to report on the structural differences between two PSTV strains which produce severe and mild disease symptoms, respectively, in tomato plants.

Materials and Methods

The two PSTV isolates were propagated in the tomato variety 'Rentita' and isolated and purified as described (4). The pathogenic isolate of PSTV originated from an inoculum kindly provided by Dr. T.O. Diener, Beltsville. Under our greenhouse conditions it produced in the tomato cultivars 'Rutgers' and 'Rentita' a severe retardation of the general plant growth (stunting) together with epinasty, rugosity, and veinal necrosis of the upper foliage.

The inoculum of the 'mild' PSTV isolate was kindly provided by Dr. K. Fernow, Ithaca, and resembles his mild strain no. 6 which he originally obtained from naturally infected potatoes in field plots of the Cornell University Potato Breeding Program. As described for some of his other mild PSTV strains (14), it produces also under our greenhouse conditions barely detectable symptoms in 'Rutgers' and 'Rentita' tomato, so it may be considered a virtually non-pathogenic PSTV isolate.

For RNA-fingerprinting and for the determination of the complete sequence of the mild PSTV isolate, 5'-phosphorylation in vitro with γ-32P-ATP and bacteriophage T4-induced polynucleotide kinase was applied exactly as described previously (5,12,15). The construction of the corresponding secondary structure model followed the principle of maximal base-pairing as previously reported (5,6).

Results and Discussion

The ribonuclease T1 and pancreatic ribonuclease fingerprints of the pathogenic and the mild PSTV isolates are shown in Fig. 1. By comparison, it turns out that the RNase T1 fingerprints of both strains (Fig. 1, A and C) differ in the location of oligonucleotides nos. 30 and 39. The nucleotide sequence of no. 30 is CAAAAAAG in the severe strain (5,15) and was established to be CAUAUAAG in the mild

Fig. 1. Fingerprints of PSTV strains causing severe (A, B) and mild (C, D) symptoms in tomato. A and C, RNase T1 fingerprints; B and D, RNase A fingerprints. The sequences of the oligonucleotides in A and B are given in ref. 5. Fingerprints were produced by electrophoresis and homochromatography as described earlier (5). All oligonucleotides were quantified and sequenced. Those differing in the two PSTV strains are marked with arrows.
isolate. The sequence of no. 39 is known to be CUUUUUCUC-UAUCUUACUUG in the severe strain (5,15) and turned out as CUUUUUCUCUAUCUUUUUG in the mild PSTV.

The RNase A fingerprints of both strains (Fig. 1, B and D) differ in that oligonucleotide no. 31 (AAAAAAGGAC) of the severe strain is replaced by oligonucleotides AAU and AAGGAC which are now comigrating with nos. 9 and 23/24, respectively, in the mild isolate. These findings are summarized in Fig. 2, which shows the previously determined (5) primary and secondary structure of the severe PSTV (top) together with that of the mild PSTV strain as newly established by sequence analysis. Thus, it becomes evident that three nucleotide changes involving the introduction of three uridines by one insertion and by two replacements of adenosines, respectively, may change a pathogenic PSTV strain to a virtually non-pathogenic isolate. Interestingly these changes are such that the total number of the 359 ribonucleotides of the PSTV molecule is not altered.

Regarding the secondary-structure model of PSTV with respect to these sequence changes, it is interesting to note that they occur in or opposite to such oligopurine sequences (i.e. nucleotides 118-122 and 307-313) which have been found to exist in all viroids studied so far (5,12). Only one of the observed sequence differences, namely that in the left half of the molecule, seems to alter the secondary structure to some degree, in that the mild PSTV strain has two more base pairs in this region than the severe strain. Therefore, the question arose whether the number of base pairs, i.e., the secondary structure around nucleotides nos. 50 and 310, could in some way determine the pathogenic response of the host plant during PSTV replication in tomato plants.

Dickson et al. (13) have analysed fingerprints of PSTV isolates of different pathogenicities after labelling in vivo with $^{125}$I. From the description of their viroid isolates and from a recent study of viroid host range and symptomatology (16), it may be inferred that even more pathogenic PSTV isolates seem to exist than the strain we have used in our investigations. According to the experience of Dickson and co-workers (13,16), the PSTV from Dr. Diener's laboratory is an.

Fig. 2. Primary and secondary structure of PSTV strains causing severe (top) and mild (bottom) symptoms in tomato. Around nucleotide no. 120, two adenosines in the severe strain have been replaced by one uridine in the mild strain, whereas in the neighbourhood of nucleotide no. 310 an adenosine of the severe strain has been replaced by a uridine, and another uridine has been inserted, in the mild strain. There are no further differences between these strains, since the mild PSTV strain was sequenced as completely as the severe strain (5). Note that the two strains differ slightly in their secondary structures, the mild strain having two additional base pairs. The nucleotide changes from the severe to the mild strain are shown separately in the boxes.
'intermediate' isolate, which could also apply to our strain from the same source. However, the interpretation of ¹²⁵I-fingerprints is difficult because RNase T₁ oligonucleotides without a cytidine and pancreatic fragments with a 3'-terminal uridine will not become labelled. In the context of correlating changes in secondary structure and pathogenicity, it is interesting that the (even more) severe isolates of Dickson et al. (13) could be interpreted as having an additional mutation in the region around nucleotides 50 and 310 as compared to the corresponding mild and intermediate strains investigated including our severe isolate. However, this interpretation is correct only if we assume that their intermediate strain and our severe PSTV strain are identical. If so, the absence of the unique GAGC in their severe isolates would result from an additional mutation in the region of nucleotides 44-47.

Considering the more dynamic aspects of PSTV structure, it is of interest that all the observed mutations are located outside those regions which have been shown to be directly involved in the formation of branched intermediates, i.e., in stable hairpins which are newly formed during the thermal denaturation of the PSTV molecule (6,17). The existence of these regions and the formation of the corresponding hairpins in several viroid species suggests that they are of functional importance (6,17). Although their relevance for certain biological properties is not yet understood, there are indications that they could act as specific signals during viroid transcription (18). With respect to the theoretical translation potential of viroids (5,8-10), it should be noted that the described mutations would change the reading frame, in that the infecting (+) RNA molecule codes for peptides or proteins, and the replacement of adenosine in position 120 by uridine would create a possible UAA terminator codon.

Approaching the problem from a more biological standpoint, it must be emphasized that pathogenicity is a complex biological property and that the expression of disease symptoms is specified by the genome of both the viroid isolate and the host plant. Thus not only do severe, intermediate, and mild viroid strains exist, but different tomato cultivars may respond with severe (cv. Rutgers), mild (cv. Rentita), or practically no symptoms (cv. Hilda 72) upon infection with one and the same viroid, although replication and accumulation is about the same in all three host plants (19). Moreover, symptom development is dependent on a threshold concentration of the viroid, as is demonstrated after inoculation with highly dilute inocula. In this case pronounced symptoms may be recognized only after an extended period or in the newly growing axillary buds after decapitation, which can be directly related to the increased viroid concentration in the corresponding tissue. Finally environmental factors such as the nature, intensity, and length of illumination and the temperature during plant growth may greatly influence the response of the host plant to viroid infection and also the intensity of viroid replication itself (20). Nevertheless, the knowledge of structural details of more viroid isolates will be necessary for definite assessment of the influence of viroid sequence and structure on the biological properties of these pathogens.
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